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Enhancing Osteoclastic Resorption for the Prevention and Treatment of Heterotopic Ossification

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14. ABSTRACT

Bone-resorbing osteoclasts normally resorb ectopic mineral in their innate immune role. Therefore, we hypothesize that: blockade of osteoclastic bone resorption is required for heterotopic bone formation and that ***lifting repression will allow resorption*** of ectopic bone in heterotopic ossification.

Purpose: to test methods to enhance osteoclast activity to reduce HO.

Scope: This work will use a mouse model of HO to test mechanisms to enhance osteoclast formation and function. Osteoclast blockade is released by antibody inhibition of OPG, the natural antagonist of RANKL. OPG knockout (ko) mice were tested for the ability to form HO.

Major findings: We have characterized a mouse model of HO by histological and histochemical analysis, X-ray, and micro-CT imaging. We show few osteoclast cells in HO bone relative to adjacent skeletal elements. We show that anti-OPG antibody treatment reduces bone formation (by micro-CT analysis), increases osteoclast numbers and serum resorption products and improves range-of-motion in the HO model (by passive ROM assay). We show that the OPG ko mouse makes little mineralized HO yet produces cartilage. Our results support our hypothesis and show that enhanced resorption may be a novel approach to treat HO.

15. SUBJECT TERMS
Heterotopic ossification, Rehabilitation, Neuromusculoskeletal Injuries, Blast trauma, Orthopaedic trauma, Osteoclast, Osteoclastic resorption

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Introduction:

The purpose of this study is to test the hypothesis that osteoclastic bone resorption is inhibited in heterotopic ossification and that if inhibition is lifted, heterotopic ossification will be decreased or reversed. The proposal consists primarily of three main experiments, addressing two different mechanisms to induce osteoclastic resorption of heterotopic bone. Using a mouse model of heterotopic ossification we will test activation of osteoclasts by treatment with exogenous RANKL, blockade of the RANKL decoy receptor OPG with antibodies, and testing the ability of OPG knockout mice to develop heterotopic ossification.

Keywords:

Heterotopic ossification, Heterotopic bone, Rehabilitation, Neuromusculoskeletal Injuries, Blast trauma, Orthopaedic trauma, HO Treatment, Osteoclast, Osteoclastic resorption.

Overall Project Summary:

Overall we have made significant progress toward proving our central hypothesis that osteoclastic resorption is blocked in HO and that release of that blockade reduces HO. We have employed the caALK2 (a.k.a, caACVR1) mouse[1] model of HO to robustly and rapidly produce heterotopic bone and to limit limb mobility. In this model, a silent copy of the hyperactive BMP receptor is activated by cre recombinase provided by a single intramuscular injection of adenovirus expressing cre (Ad-cre). We have characterized the HO produced histologically and histochemically and show that it occurs via an endochondral sequence showing residual cartilage and calcified cartilage. We also note the presence of vasculature, adipose tissue, bone (woven and lamellar), and marrow in a disordered organization in the HO produced, which is similar in composition and organization to human HO. We have imaged the HO produced by micro-computed tomography (micro-CT) and have identified a suitable approach to quantification of HO bone. Instead of trying to tease out heterotopic bone from orthotopic bone in the micro-CT scans, we measure total mineral volume in the injected hindlimb, from hip to ankle, and compared to bone volume in the un-injected contralateral limb. This method allows assessment of treatment effects on other skeletal elements and acts as an internal control for animals that might start with different skeletal bone densities.

Having identified suitable function blocking anti-OPG antibodies we tested their ability to release our hypothesized block of osteoclastic resorption in HO and to induce osteoclast formation and resorption of HO. Anti-OPG treatment was shown histologically to increase osteoclast numbers in HO bone over IgG controls. Anti-OPG treatment was shown to increase serum TRAP as a marker of osteoclast formation over IgG controls. Anti-OPG treatment was shown to increase serum collagen crosslinks over IgG injected controls as a measure of bone resorption. We show that over a range of Ad-cre doses, I.P. anti-OPG administration improves range of motion relative to IgG injected controls in a statistically significant manner. Anti-OPG treatment decreases the total volume of bone present relative to IgG controls. Anti-OPG does decrease bone volume in the contralateral limb but not as much as in HO bone, an effect we believe is due to the high OPG expression in HO bone, which renders it a selective target for anti-OPG therapy. Finally, we show that HO formation is significantly reduced or non-existent in OPG knockout mice. We are currently increasing OPG KO animal numbers and testing rescue of HO by alendronate administration for publication.

These results support our hypothesis that osteoclastic bone resorption is inhibited in HO and indicate that pro-resorptive therapy may be a useful approach for treatment of HO.

Key Research Accomplishments:

Task 1. Breed sufficient caACVR1 (aka caAKL2) conditional transgenic mice and controls for *in vivo* experiments.
Months (4-24): Breeding ACVR1 animals
Progress: completed
We continue to maintain a breeding colony of caACVR1 animals for use as needed for experimental replicates and collect any additional data required for publication.

Task 2. Breed sufficient OPG-/- mice and controls and cross in the caACVR1 transgene for *in vivo* experiments.
Months (6 – 30): Breeding mice OPG-/-; ACVR1+/+ animals
Progress: completed
The OPG-/- animals originally bred poorly since they often consumed their offspring. In addition, we find that the OPG-/- animals are extremely fragile due to very low bone density and these animals were difficult to breed. The caACVR1 gene was bred to homozygosity on the OPG-/- background since we found that results using the model were significantly more consistent with 2X caACVR1 transgenes. The caACVR1+/+ animals could only be identified by backcross to wildtype which required more breeding than originally anticipated.

Task 3. Produce sufficient adenoviral-Cre (ad-Cre) and control virus (ad-GFP) at high titer for induction of HO in the mouse model.
Months (1 – 30)
Progress: completed
Ad-Cre adenovirus routinely produces strong HO in the homozygous caACVR1 animals and it was required for all experiments using the caACVR1 model.

Task 4. Test RANKL effects on heterotopic ossification development.
Months (6 – 24).
Preliminary experiments with RANKL produced negligible positive effects and were reported as such by other investigators[1]. Due to the positive results demonstrated using the anti-OPG approach (***Task 5***), efforts were concentrated in this area.

Task 5. Test anti-OPG antibodies for function blocking in the mouse model in vitro.
Months (3 – 12)
Progress: completed
Work on Task 5 was completed in Year 1 as we have tested and identified 2 commercially available goat anti-OPG polyclonal antibody preparations that show function blocking of OPG in culture of RAW264.7 cells with RANKL (R&D Systems cat# AF459, and Sigma cat# O1139) (**Figures 1A and 1B**). Both commercial preps function similarly as the dose required to reverse OPG blockade of osteoclastogenesis is essentially the same.

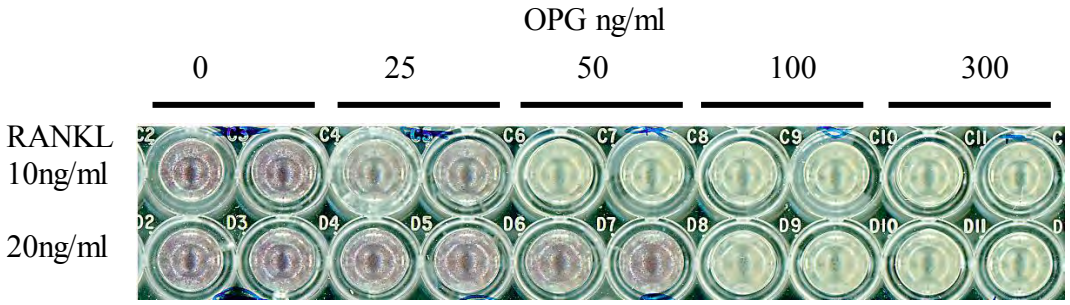


Figure 1A. An example of titration of OPG for inhibition of RANKL-induced osteoclast formation by RAW264.7 cells. TRAP stain (violet) for osteoclast formation. Multinuclear cell formation closely follows TRAP expression (micrographic data not shown).

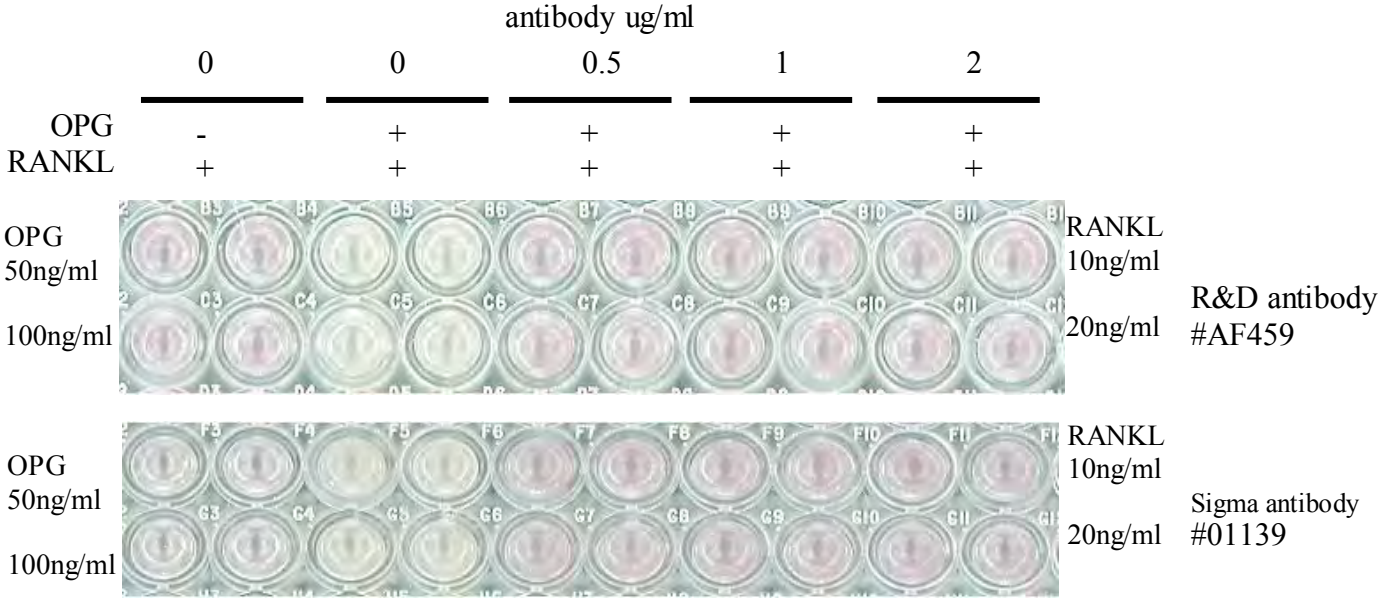


Figure 1B. Both Sigma and R&D anti-OPG antibodies block OPG inhibition of RANKL-induced osteoclast formation by RAW264.7 cells. TRAP stain (violet) for osteoclast formation. Both preps completely reverse 100ng/ml OPG blockade when added at 0.5ug/ml (arrows).

Task 6. Test anti-OPG effects on heterotopic ossification development (using mice produced in ***Task 1*** and ad-Cre from ***Task 3***)
Months (12 - 24)
Progress: completed

Having established breeding colonies, the mouse model, passive range-of-motion assay, histological procedures for H&E, toluidine blue, and TRAP staining, and having determined the best approach for micro-CT analysis of HO, we have established and characterized the caALK2 HO model (**Figures 2A & B and Figures 3A & B**). We have adopted a sensitive passive range-of-motion assay based on one presented by Paul Yu[2] (**Figure 2**). In unaffected animals, hind limbs can be gently moved parallel to and touching the abdomen (ROM=0). Impaired mobility is detected when the limb can move less than 20^0, relative the body, indicating a small decrease in mobility (ROM=1). Moderate impairment is noted if the limb can move between 20^0 and 45^0 relative to the body (ROM=2). Severe impairment is noted if the limb can only move 45^0 to 90^0 to the body side. At this point knee joint mobility begins to be affected (ROM=3). Mobility of the knee becomes impaired and hip joint motion begins to stiffen. The limb cannot move between 90^0 and 135^0 relative to the body (ROM=4). Animals are sacrificed when no mobility is noted in the ankle, knee, or hip and the limb cannot move between 135^0 and 180^0 relative to the body (ROM=5). (**Fig 1B**) Following I.M. injection of adenoviral-Cre into the right hind limb of caAlk2 mice, loss of limb mobility occurs over a rapid and reproducible, 14 day time course.

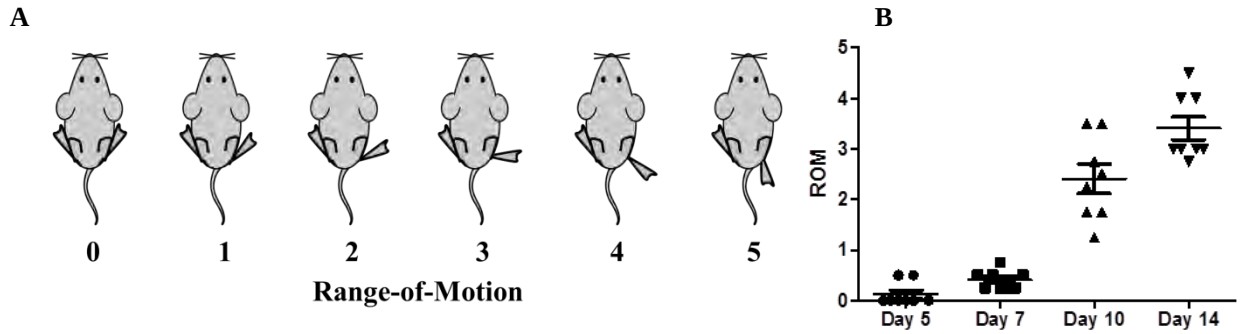


Figure 2. Passive Range-of-Motion (ROM) assay (**A**) and time course (**B**) of the caALK2 HO model.

Histological analysis was performed on HO tissues to characterize the cell and tissue reactions.

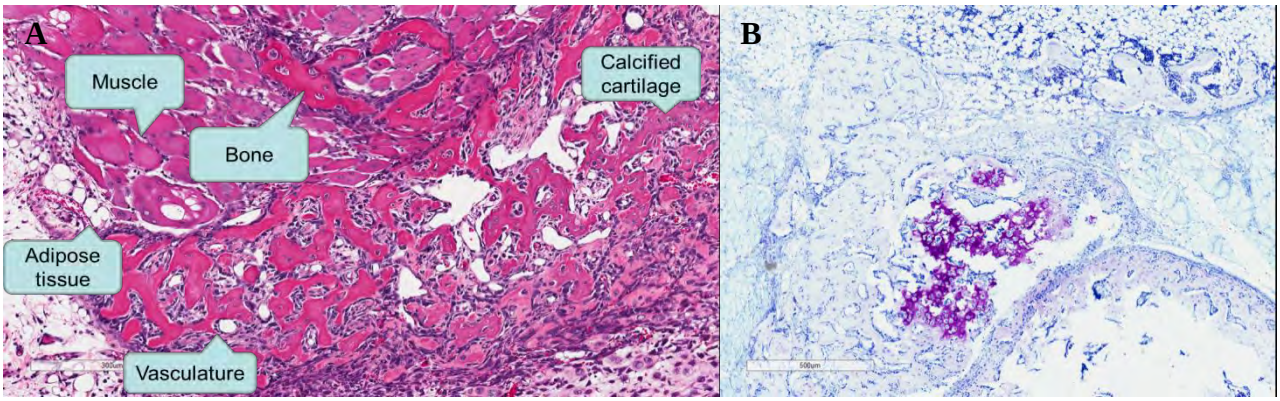


Figure 3. Characterization of the caALK2 model of HO. (**A**) H&E stained section of HO displaying the disorganized structure of HO and presence of calcified cartilage, vasculature, adipose tissue, muscle and bone. (**B**) Toluidine blue stained section showing the presence of cartilage (violet color) indicative of the endochondral formation sequence of HO.

Sections of HO bone, including normal skeletal elements (orthotopic bone), were stained for the osteoclast marker tartrate-resistant acid phosphatase (TRAP) (**Figure 4**).

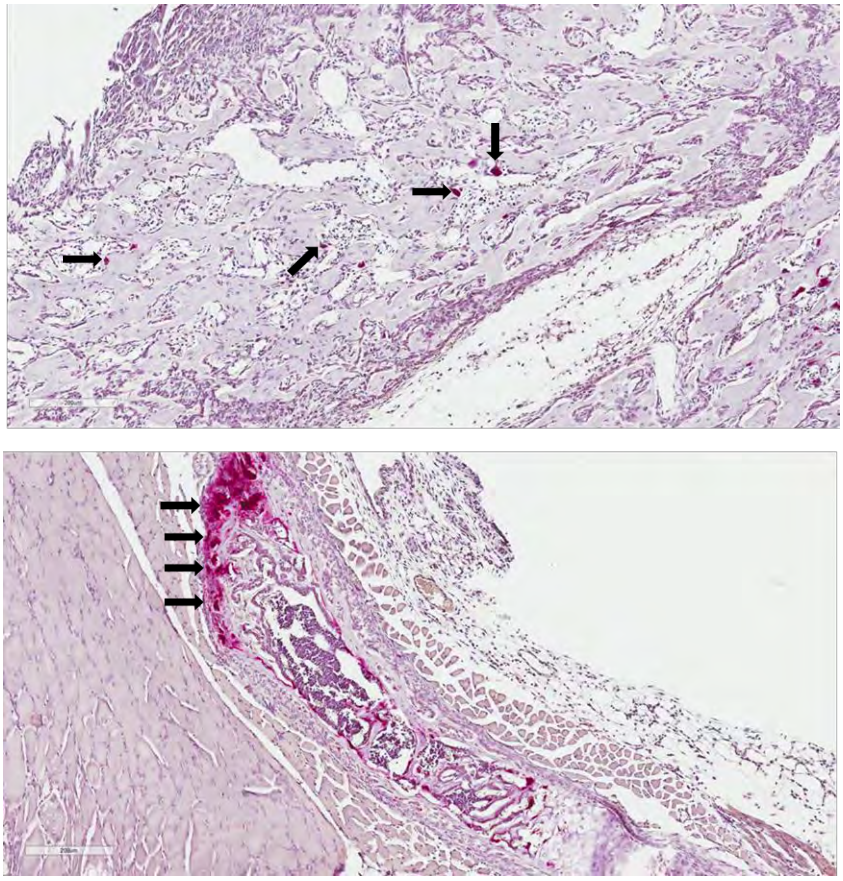


Figure 4. There are few osteoclasts in HO bone. Sections of experimental HO, containing both HO and skeletal bone, were stained for the osteoclast marker tartrate-resistant acid phosphatase (TRAP). Few TRAP-positive (red) osteoclast cells are seen in the heterotopic bone (top panel, arrows). Numerous TRAP-positive osteoclast cells are seen in growing skeletal bone in the same histological section (bottom panel).

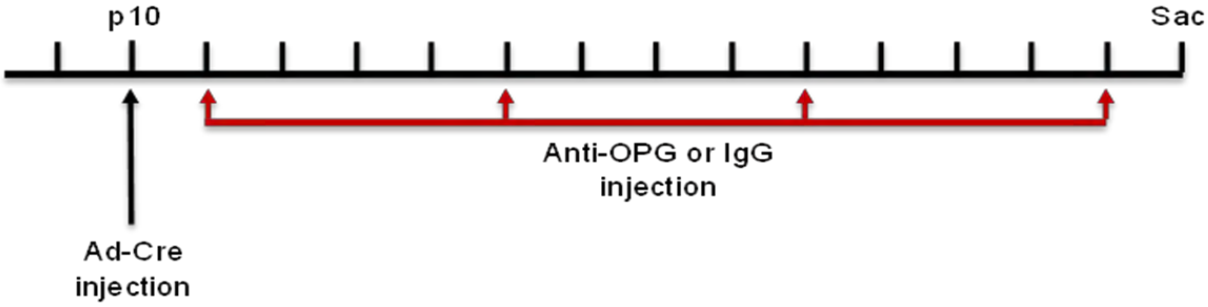


Figure 5. Anti-OPG treatment. Animals were injected I.P with goat anti-OPG, as identified and tested in Task 5, at 1g/kg body weight or control IgG every 4 days

We find that I.P. injection with function-blocking goat polyclonal anti-OPG increases osteoclast numbers in HO bone. **Figure 6** shows TRAP stained osteoclasts in two IgG injected (control) and 2 anti-OPG (experimental) animals. Serum TRAP, as a marker of osteoclastogenesis is significantly increased with anti-OPG treatment over IgG treated controls (**Figure 7A**). Serum Collagen C-terminal telopeptide (CTx) as a marker for osteoclastic bone resorption is also increased by anti-OPG treatment over IgG control (**Figure 7B**).

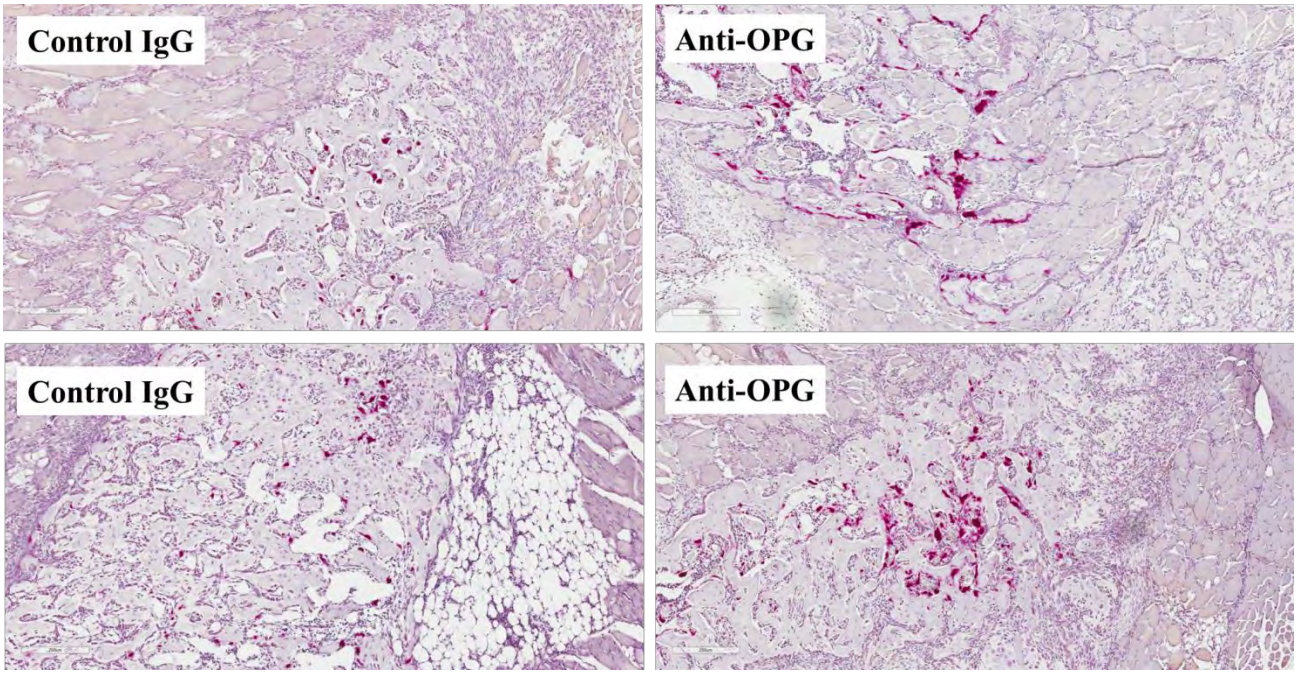


Figure 6. Systemic treatment with anti-OPG function-blocking antibodies increases osteoclast numbers in HO bone. TRAP stain for osteoclast cells (red colored cells) in HO from 4 different individuals. Treatment with polyclonal anti-OPG antibodies (IP injection) leads to an increase in osteoclast numbers in HO (right panels) relative to control IgG treated animals (left panels).

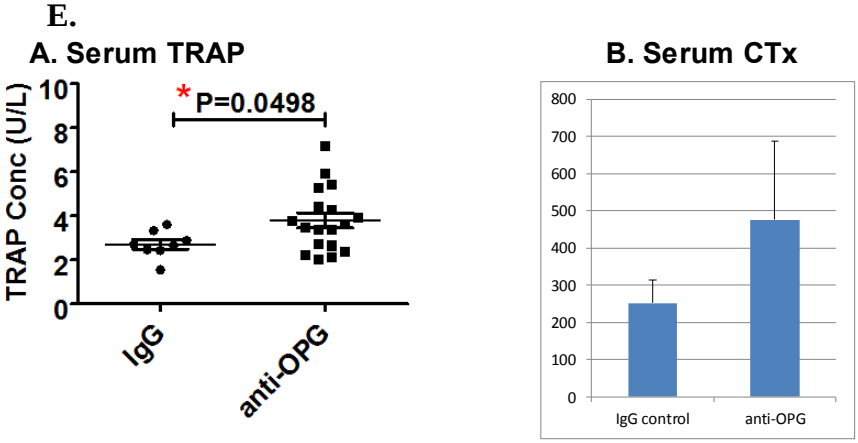
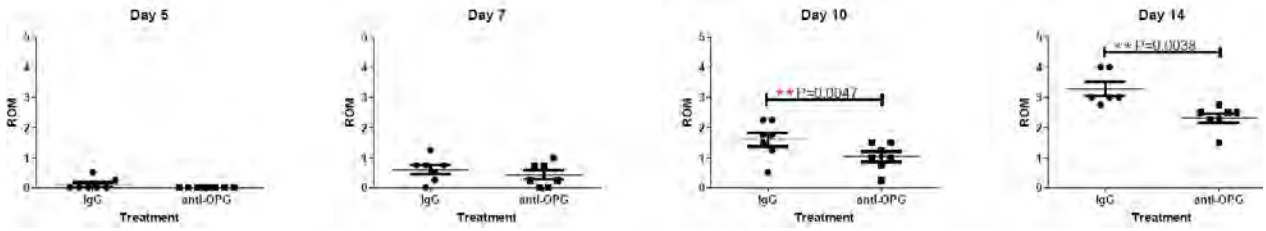


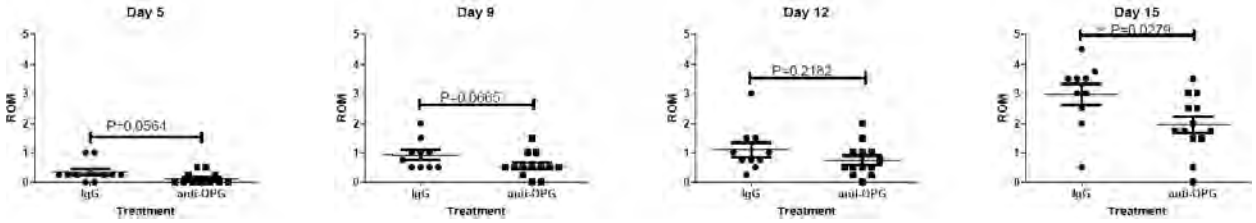
Figure 7. Systemic treatment with anti-OPG function-blocking antibodies increases serum TRAP and collagen crosslink levels. I.P. injection with polyclonal anti-OPG antibodies leads to an increase in serum TRAP5b levels, which were significantly increased in anti-OPG treated animals over control IgG treated animals (**A**). Serum Collagen crosslinks (CTx) were similarly increased by anti-OPG treatment.

To determine if anti-OPG treatment had an effect on joint mobility, passive range-of-motion assays (**Figure 2**) were performed, shown in **Figures 8A**, **8B**, and **8C**.

A. 2.4 X 10^8 pfu Ad-Cre, 1 μg/g (α-OPG or IgG/Body Weight)



B. 4.8 X 10^8 pfu Ad-Cre, 1 μg/g (α-OPG or IgG/Body Weight)



C. 9.6 X 10^8 pfu Ad-Cre, 1 μg/g (α-OPG or IgG/Body Weight)

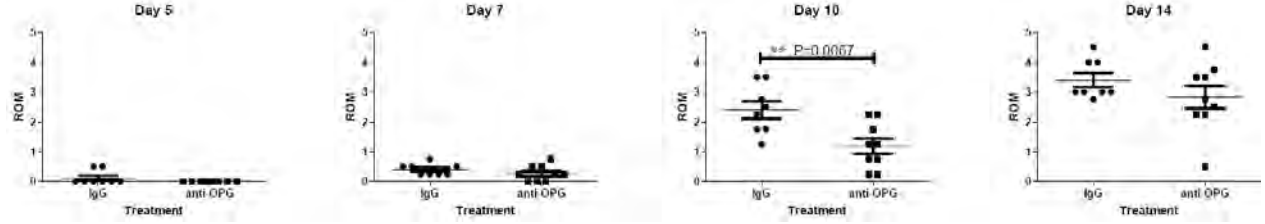


Figure 8. Treatment with function-blocking anti-OPG antibodies improves mobility. Preliminary experiments indicated that systemic treatment with anti-OPG antibodies (IP injection) resulted in an increase in mobility in treated animals relative to control IgG injected animals. In order to optimize results, we tested a range of doses of adenoviral-Cre at our standard dose (**B**) (1X = 4.8 X 108 pfu), a double dose (**C**) (2X = 9.6 X 108 pfu), and a half dose (**A**) (0.5X = 2.4 X 108 pfu), keeping anti-OPG treatment constant. We find that all adenoviral-Cre doses, anti-OPG treatments resulted in improved mobility and the statistically significant differences were found with the lowest dose of adenoviral-Cre tested (A), with significant improvement at days 10 and 14. (* $p<0.05$, ** $p<0.01$ respectively)

Analysis of data from μCT scanning (representatives shown in **Figures 9A-D**) was performed. Using a pixel size of 13.045 um, an upper grey threshold of 255 and lower grey threshold of 70, and again with the lower grey threshold of 90.

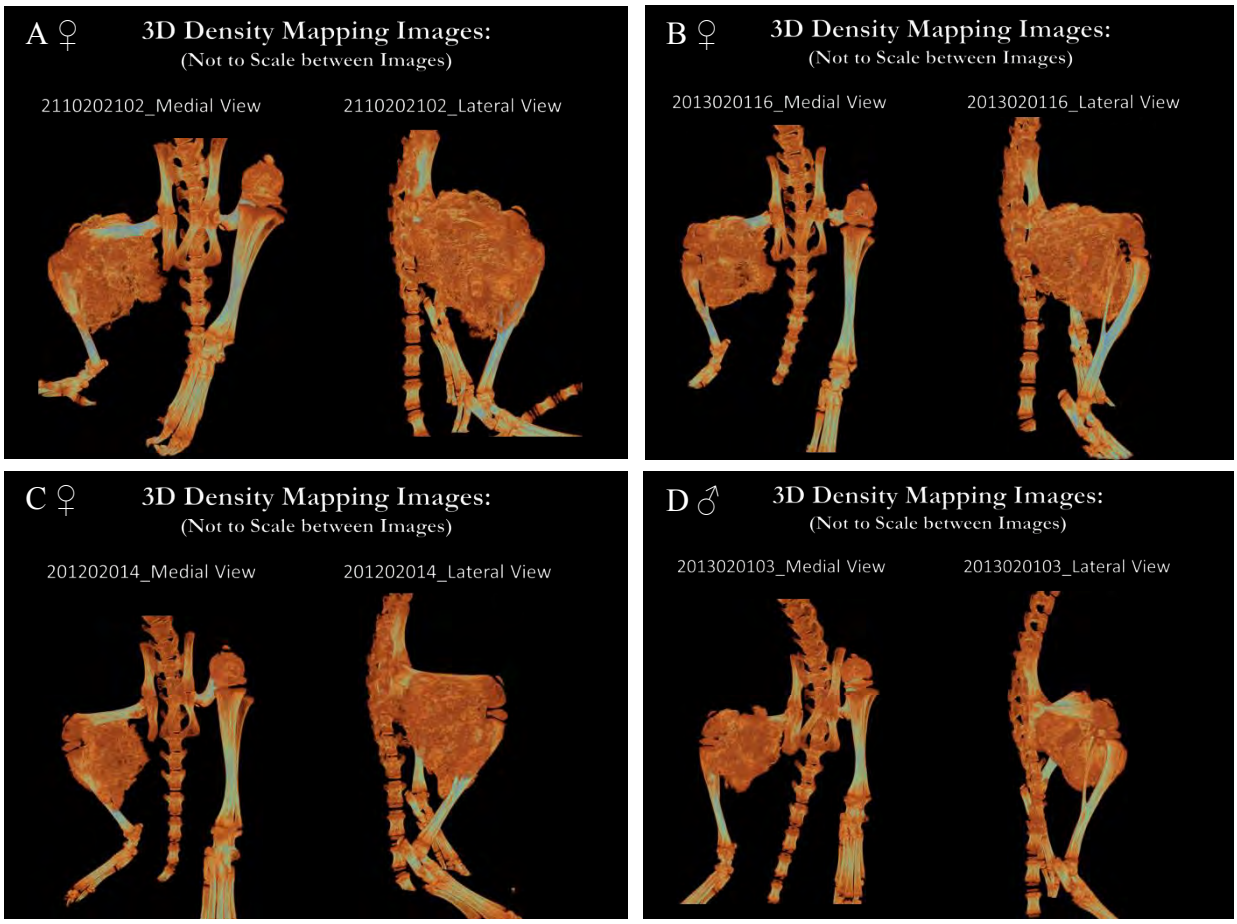


Figure 9. Micro-CT imaging of anti-OPG treated animal HO and IgG treated controls. 3D density mapping images of HO. Representative micro-CT images of 2 control IgG treated animals (**A** and **B**) and two anti-OPG treated animals (**C** and **D**). Three animals are female (**A**, **B**, and **C**) and one is a male (**D**). Images are presented in a lateral view and a medial view for each. Anti-OPG treatment leads to an apparent decrease in HO formed (**C** and **D**) relative to controls (**A** and **B**).

The following calculations were made using 3D data:

Tissue volume, Bone volume, Percent bone volume, Tissue surface, Bone surface, Intersection surface, Bone surface/volume ratio ,Bone surface density, Trabecular pattern factor, Centroid (x), Centroid (y),Centroid (z)

The following calculations were made using 2D data with both a lower threshold of 70 and with a lower threshold of 90:

Tissue volume, Bone volume, Percent bone volume, Tissue surface, Peripheral tissue surface, Bone surface, Bone surface/volume ratio, Mean crossectional tissue area, Mean total crossectional tissue perimeter, Mean total crossectional bone area, Mean total crossectional bone perimeter, Mean number of objects per slice, Average object area per slice, Average object area-equivalent circle diameter per slice, Trabecular thickness (plate model), Trabecular separation (plate model), Trabecular number (plate model), Trabecular diameter (rod model), Trabecular separation (rod model), Trabecular number (rod model), Mean trabecular pattern factor, Closed porosity (percent).

In order to quantify HO formed and to measure effects of anti-OPG treatment, we compared micro-CT parameters described above from control (left) hind limbs to limbs in which HO was induced (right). Using this approach, ectopic bone can be quantified by subtracting bone in the control limbs. Additionally, treatment effects on HO can be compared to the systemic effects of treatment by comparing to bone at a distant site (Left control limb) between treatment and control groups. Some of the relevant comparisons are presented graphically below (**Figures 10-12**).

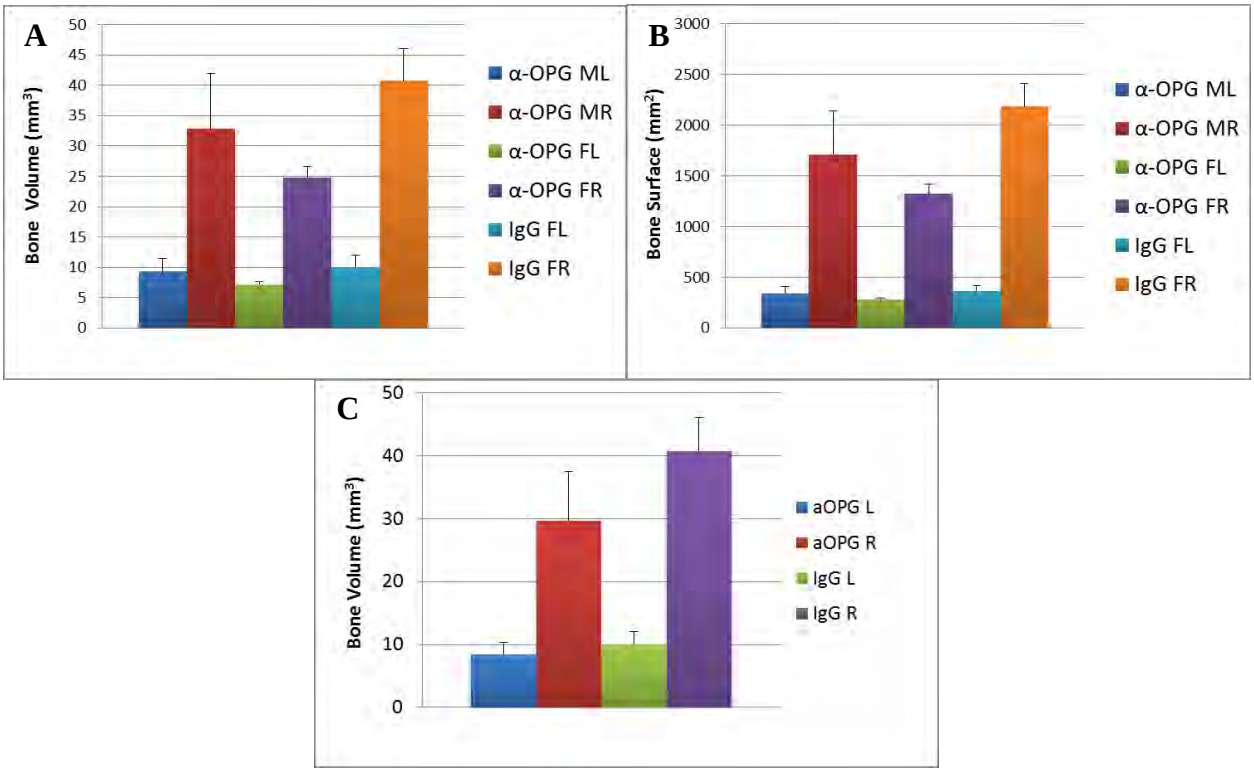


Figure 7. Figure 8. Micro-CT analysis of HO with OPG treatment; 3D measurements. Bone volume (mm^3) (**A**) in right, HO limbs, is reduced in anti-OPG treated male (αOPG MR) and female animals (αOPG FR) compared to HO in IgG treated control female animals (IgG FR). Bone volume in left, control, limbs of anti-OPG treated animals (αOPG ML and αOPG FL) is reduced as well but to a lesser extent. Bone surface measurements (mm^2) (**B**) parallel those of bone density with anti-OPG treatment decreasing HO relative to IgG treated controls and relative to bone surface in contralateral limbs. The combined results (M+F) (**C**) indicate a nearly statistically significant difference ($p<0.055$) between treatment (aOPG R) and control groups (IgG R) for bone volume in HO. A larger sample number has resulted in statistical significance.

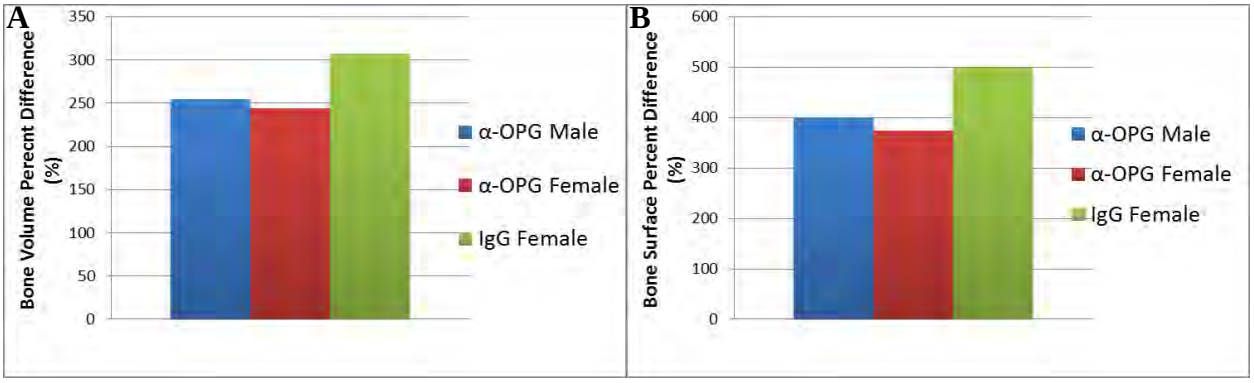


Figure 11. **Percent difference in bone volume**. The percent difference (%) in bone volume between Left and Right limbs (HO to unaffected control limbs) (**A**) is less in anti-OPG treated males and females compared to IgG treated controls. The percent difference in bone surface (**B**) parallels the bone volume results.. Systemic treatment with anti-OPG function-blocking antibodies increases serum TRAP and collagen crosslink levels

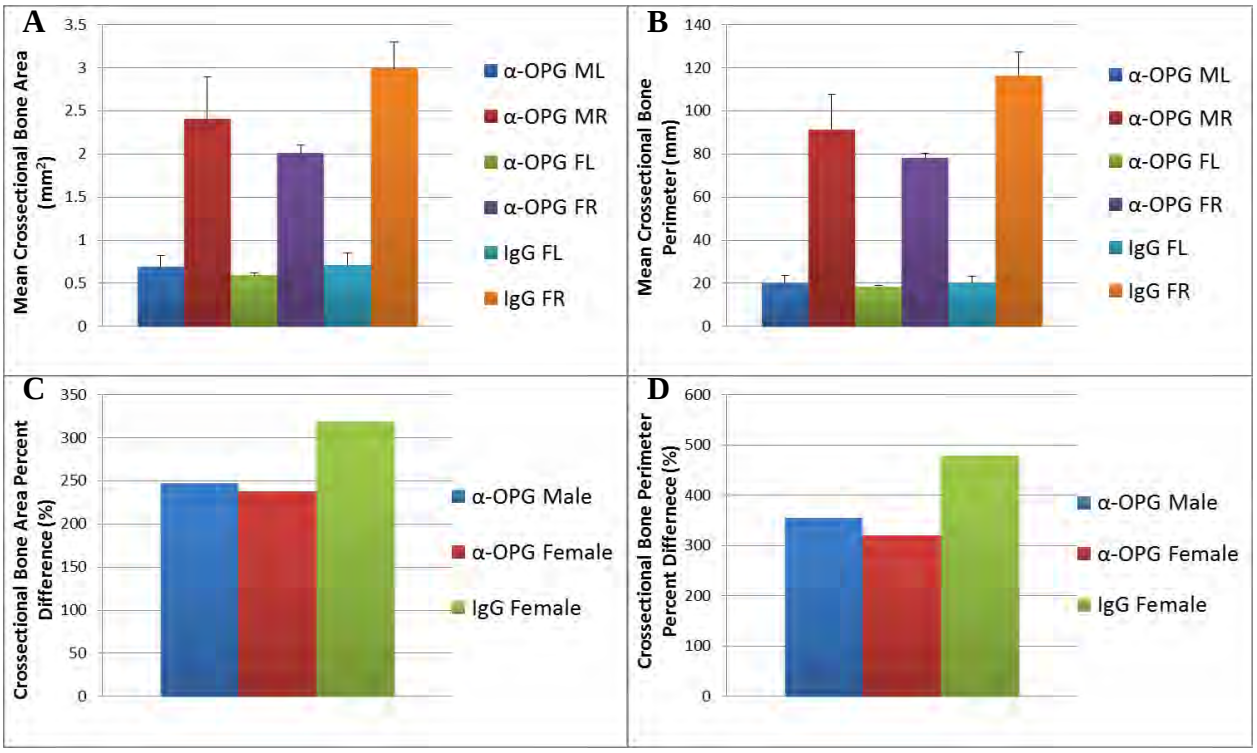


Figure 12. Micro-CT analysis of HO with 2D measurements. Analysis of 2D parameters reveals similar relationships between treatment and control groups. Mean crossectional bone area (**A**) shows a decrease with treatment in male and female groups (aOPG MR and aOPG FR) compared to control HO (IgG FR). Mean crossectional bone perimeter (**B**) shows a similar relationship with treatment reducing bone perimeter in HO limbs. Fold differences between left and right limbs for each treatment and control group (**C** and **D**) show a decrease in the 2D parameters with anti-OPG treatment.

Task 7. Test HO development in OPG-/- mouse background (using mice produced in ***Task 1*** and ***Task 2***, with ad-Cre from ***Task 3***).
Progress: near complete for publication

The caALK2 transgene was bred onto the OPG-/- background and the transgene was bred to homozygosity (caALK2 +/+) as determined by testcross, as for the caALK2 mice. HO was induced by Ad-cre injection exactly as performed with the caALK2 mice. The passive range-of-motion assay was performed and slight impingement of motion was detected (ave. =1.5) for the caALK2; OPG-/- animals at 14 days, compared to 4.5 for caALK2. Micro-CT imaging shows little to no mineral formed in the caALK2; OPG-/- animals (**Figures 13A and B**).

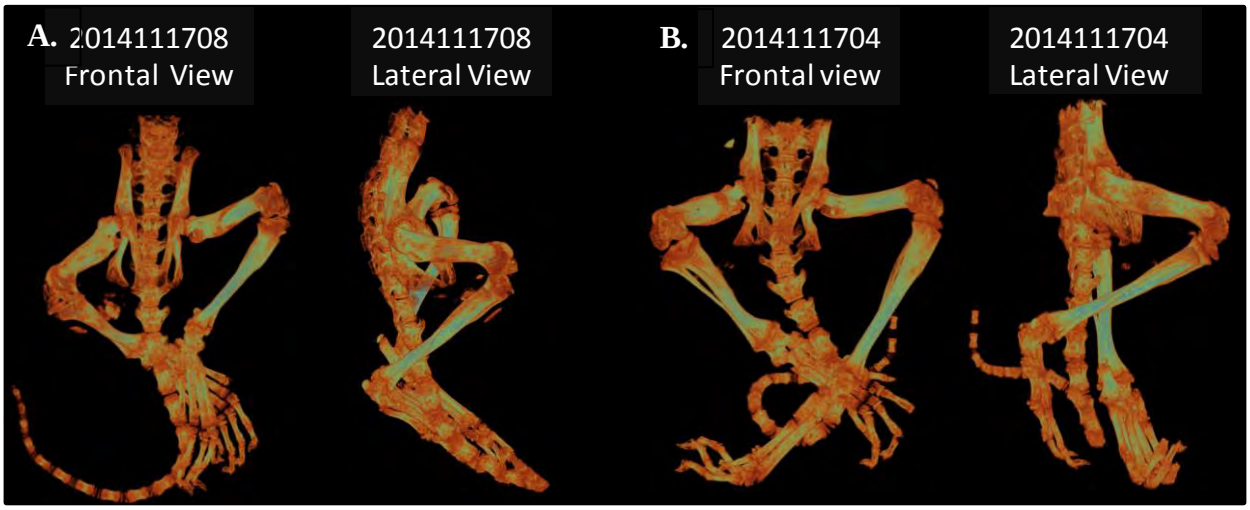
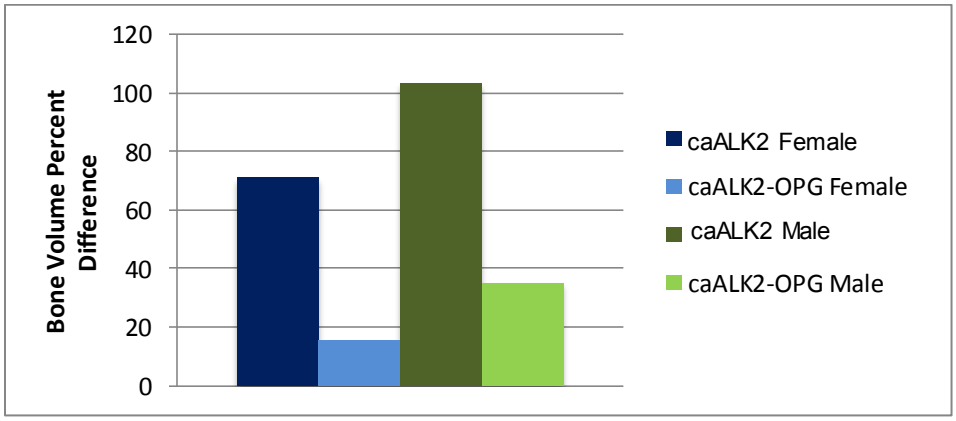


Figure 13. Representative micro-CT images of HO in OPG-/- mice. 3D density mapping images of female (**A.**) and male (**B**.) images caALK2; OPG-/- mice 14 days following Ad-cre injection. Small wispy mineral deposits can be seen in the right hindlimbs where caALK2 animals routinely produce large HO deposits.

Quantification of micro-CT results shows a significant decrease in HO production by caALK2;OPG-/- animals relative to caALK2 controls (**Figure 14,** below).



We had originally proposed to test reversal of any OPG knockout HO phenotype with administration of exogenous OPG however a better strategy has presented itself in treatment with alendronate. If the OPG-/- HO phenotype is solely dependent upon enhanced resorption the blockade of resorption from outside of the RANK/RANKL/OPG axis should rescue the HO phenotype. This approach eliminates any other OPG signaling pathways since OPG has multiple partners (e.g. TRAIL blockade) and multiple functions (e.g. immune).

We are currently administering alendronate to caALK2;OPG-/- animals and should have confirmation for publication soon.

Key Research Accomplishments:

- We have demonstrated a statistically significant improvement in range-of-motion with systemic pro-resorptive, anti-OPG, treatment in a mouse model of HO.
- We have demonstrated an increase in osteoclast numbers with pro-resortpive, anti-OPG, treatment in a mouse model of HO.
- We have demonstrated a decrease in HO bone with pro-resorptive, anti-OPG, treatment in a mouse model of HO.
- We have demonstrated a significant defect in HO formation in the OPG-/- mouse background.

Conclusions:

We have generated data demonstrating statistically significant reduction of experimental HO by treatment with anti-OPG antibodies. Importantly this is associated with an increase in osteoclast numbers and an increase in serum markers of resorption. These results confirm our hypothesis that bone resorption is blocked in HO. We have established that in the animal model, high OPG expression in HO makes it a selective target for anti-OPG pro-resorptive therapy. Accordingly, bone loss at distant skeletal sites (contralateral limbs), was minimal and significantly less than bone loss at HO sites.

Completion of this work establishes a new molecular mechanism involved in HO formation and supports the further testing of novel pro-resorptive treatments for HO.

Publications, Abstracts and Presentations:

Seminar Presentations:

1. McHugh, KP
   Osteoclasts, Osteoblasts & HO
   Translational Research Seminar
   University of Florida College of Dentistry
   Gainesville, FL
   October 25, 2011

2. McHugh, KP
   Treatment of Heterotopic Ossification by Enhancing Bone Resorption
   Grand Rounds
   Department of Orthopaedic Surgery
   University of Texas Health Science Center
   San Antonio, TX
   September 16, 2013.

Abstracts and Podium Presentations:

1. Xue S, Fajardo RJ, McHugh KP
   Enhancing Osteoclastic Resorption for the Prevention and Treatment of Heterotopic Ossification
   Poster
   Gordon Research Conference; Bones and Teeth
   Galveston, TX
   January 26-31, 2014

2. Xue S, Fajardo RJ, McHugh KP
   Pro-resorptive Therapy for Heterotopic Ossification.
   Oral Poster, Plenary Poster
   American Society for Bone and Mineral Research
   Houston, TX
   September 12-15, 2014

3. McHugh KP
   A Pro-Resorptive Approach to Treatment of Heterotopic Ossification.
   Podium Presentation; Selected Poster Abstract
   Extremity War Injuries X: Return to Health and Function
   Washington, DC
   January 26-28, 2015.

4. Xue S, Fajardo RJ, McHugh KP.
   Development of Pro-resorptive Therapy for the Treatment of Heterotopic Ossification.
   Oral Paper
   Orthopaedic Research Society
   Las Vegas, NV
   March 28-31, 2015.

5. Xue S, Schmitz J, Fajardo RJ, McHugh KP.
Osteoprotegerin (OPG) Expression is Required for Mineralization of Heterotopic Bone manuscript in prep

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P.I.: McHugh KP
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Title: "Development of Targeted Pro-Resorptive Therapy for the Treatment of Heterotopic Ossification"

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P.I.: McHugh, KP
NIH/NIAMS R21
Title: "Pro-resorptive Approaches for Treatment of Heterotopic Ossification"

Inventions, Patents and Licenses:

None

References:

1. Fukuda T, et. al.,
Generation of a Mouse With Conditionally Activated Signaling Through the BMP Receptor, ALK2
Genesis 2006, 44;159

2. Frederick P. Heinzel and Xin Li
Pathogenesis of Heterotopic Ossification in Traumatic Brain Injury
USAMRMC Grant # W81XWH-08-2-0065
Annual Report 2009

3. Paul Yu et. al.
BMP type I receptor inhibition reduces heterotopic ossification
Nature Medicine, Vol. 14(12), 2008

Appendices:

1. Abstract submitted to Gordon Conference: Bones and Teeth 2014
2. Abstract submitted to ASBMR 2014
3. Abstract submitted to EWI X 2015
4. Abstract submitted to ORS 2015

Gordon Research Conference; Bones and Teeth, Galveston, TX, January 26-31, 2014

Enhancing Osteoclastic Resorption for the Prevention and Treatment of Heterotopic Ossification

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Abstract:

Heterotopic ossification (HO) is the formation of bone in non-osseous tissues. HO is a frequent complication of orthopaedic surgery, especially following blast-trauma, with two thirds of these patients developing painful and debilitating HO. There are currently no effective treatments for HO other than surgical excision of the ossified tissue which is an extremely difficult and morbid procedure. Therefore, there is a significant unmet need for effective and non-invasive treatment of HO.

Bone metabolism is regulated by the local expression and ratio of the osteoclast inducer Receptor Activator of Nuclear Factor B (RANKL) to the native osteoclast inhibitor osteoprotegerin (OPG). Osteoblasts express high RANKL/OPG early in their differentiation thus driving resorption, while mature, mineralizing, osteoblasts express low RANKL/OPG. We hypothesize that OPG expression is high in HO, blocking osteoclast formation, favoring maintenance of heterotopic bone. We propose that releasing the OPG blockade of osteoclast formation by systemic administration of anti-OPG function-blocking antibodies will allow osteoclast formation and prevent the development of HO.

We employed a mouse model of HO in which transgenic mice harbor an inducible constitutively-active ALK2 receptor construct (caALK2)(an R220A mutant of the BMPRII receptor ALK2). Expression of the caALK2 gene is induced by IM injection of Cre-recombinase delivered by an adenoviral vector (Ad-Cre). Following IM injection of Ad-Cre, animals reproducibly develop significant ectopic bone at the site of injection over a rapid 14 day time course. Systemic treatment is by IP injection of anti-OPG antibody (10ug on days 2, 6, 10). Commercially available anti-OPG antibodies were tested for their ability to block OPG inhibition of in vitro osteoclastogenesis using RAW264.7 cells cultured with 20ng/ml RANKL. HO production was demonstrated over time by a "range-of-motion" assay as described by Paul Yu et al (NatMed 2008). MicroCT was used to quantify HO formation. Histology was used to characterize the HO formed and to identify TRAP positive osteoclasts.

We find that systemic treatment with an inhibitory antibody against OPG results in a statistically significant reduction of mineralized tissue in HO with a concomitant and statistically significant improvement in the range of motion relative to IgG treated controls. The OPG blockade results in an increase in osteoclast numbers in ectopic bone. Importantly, the bone loss at ectopic sites appears to be greater than loss at native skeletal sites in the same animal.

Our studies indicate that osteoclastic resorption is likely inhibited in HO by OPG expression and that if OPG function is blocked, HO is reduced. Therefore, pro-resorptive therapies for HO may have a significant impact on the prevention and treatment of HO, to relieve this painful and debilitating orthopaedic complication.

American Society for Bone and Mineral Research, Houston, TX, September 12-15, 2014

Pro-Resorptive Therapy for Heterotopic Ossification

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Abstract:

Heterotopic ossification (HO) is the formation of bone in non-osseous tissues. HO is a frequent complication of orthopaedic surgery, especially following blast-trauma, with two thirds of patients developing painful and debilitating HO. There are currently no effective treatments for HO other than surgical excision of the ossified tissue which is an extremely difficult and involved procedure. Therefore, there is a significant unmet need for effective and non-invasive treatments of HO.

Bone metabolism is regulated by the local expression and ratio of the osteoclast (OC) inducer RANKL to the native OC inhibitor osteoprotegerin (OPG). We show that OPG expression is higher and OC numbers are much lower in HO bone, relative to native skeletal sites. We hypothesize that high OPG expression is required for HO, blocking OC formation and favoring maintenance of heterotopic bone. We propose that releasing the OPG blockade of OC formation, with function-blocking anti-OPG antibodies, will allow OC formation and prevent the development of HO. Importantly, due to the high OPG expression, HO will be a selective target of this pro-resorptive approach.

We employed a mouse model of HO in which transgenic mice harbor a Cre-inducible constitutively-active ALK2 receptor construct (caALK2)(a R220A mutant of the BMP receptor ALK2). Expression of the caALK2 gene is induced by intramuscular injection of adenoviral Cre (Ad-Cre). Following injection of Ad-Cre, animals reproducibly develop significant ectopic bone at the site of injection over a rapid 14 day time course. Systemic treatment is by intraperitoneal injection of anti-OPG antibody. HO production was demonstrated over time by a range-of-motion (ROM). MicroCT was used to quantify HO formation and contralateral native bone volume. Histology was used to characterize the HO and to identify TRAP positive OCs.

Systemic treatment with an inhibitory antibody against OPG results in an increase in OCs in ectopic bone. Statistically significant reduction of mineralized tissue in HO was seen with a concomitant and statistically significant improvement in ROM, compared to IgG treated controls. Notably, bone loss seen at ectopic sites was greater than loss at native skeletal sites in the same animal.

These studies indicate that osteoclastic resorption is likely inhibited in HO by OPG expression and if OPG function is blocked, HO is reduced. Therefore, novel pro-resorptive therapies for HO may have a significant impact on the treatment of HO.

POSTER ABSTRACT

Title	**A Pro-Resorptive Approach to Treatment of Heterotopic Ossification**
Presenter	**Kevin P. McHugh, PhD**
Poster #	**10**

Heterotopic ossification (HO) is the formation of bone in non-osseous tissues. HO is often associated with trauma and is frequently seen following blast-trauma with two thirds of patients developing painful and debilitating HO. There are currently no effective treatments for HO other than surgical excision of the ossified tissue which is an extremely difficult and involved procedure. There is therefore a significant unmet need for effective and non-invasive treatment for HO.

Bone metabolism is regulated by the local expression ratio of the osteoclast inducer Receptor Activator of Nuclear Factor kB Ligand (RANKL) to the native osteoclast inhibitor osteoprotegerin (OPG). We hypothesize that OPG expression is high in HO, blocking osteoclast formation and favoring maintenance of heterotopic bone. We propose that releasing the OPG blockade of osteoclast formation by systemic administration of anti-OPG function-blocking antibodies will allow osteoclast formation and prevent the development of HO.

Importantly, due to the high OPG expression, HO will be a selective target of this pro-resorptive approach. We employed a mouse model of HO in which transgenic mice harbor a Cre-inducible constitutively-active ALK2 receptor construct (caALK2)(a Q207D mutant of the BMP receptor ALK2). Expression of the caALK2 gene is induced by intramuscular injection of adenoviral Cre (Ad-Cre). Following injection of Ad-Cre, animals reproducibly develop significant ectopic bone at the site of injection over a rapid 14 day time course. Systemic treatment is by intraperitoneal injection of anti-OPG antibody. HO production was demonstrated over time by a range-of-motion (ROM). MicroCT was used to quantify HO formation and contralateral native bone volume. Histology was used to characterize the HO and to identify TRAP positive OCs.

We show that HO bone contains few osteoclast cells compared to long bones and that HO tissues express high OPG as compared to normal skeletal sites. Systemic treatment with the inhibitory polyclonal antibody against OPG results in an increase in osteoclast numbers in HO bone with an increase in serum TRAPC5b ($p<0.05$). Systemic treatment with anti-OPG also resulted in a statistically significant reduction of mineralized tissue in HO ($p=0.05$) with a concomitant and statistically significant improvement in ROM at day 14 ($p=0.0038$), compared to IgG treated controls. Notably, bone loss seen in anti-OPG treated animals was greater at ectopic sites than loss at native skeletal sites.

These studies indicate that osteoclastic resorption is likely inhibited by OPG expression in HO and if OPG function is blocked, HO is reduced. These studies demonstrate that enhanced resorption can decrease HO formation and our future studies will test pro-resorptive approaches on mature human HO.

Development of Pro-Resorptive Therapy for the Treatment of Heterotopic Ossification

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Disclosures: **S. Xue:** None. **R.J. Fajardo:** None. **K.P. McHugh:** None.

Introduction: Heterotopic ossification (HO) is the formation of bone in non-osseous tissues. HO is often associated with trauma and frequently a significant proportion of patients develop painful and debilitating HO. There are currently no effective treatments for HO other than surgical excision of the ossified tissue which is an extremely difficult and involved procedure. Therefore there is a significant unmet need for effective and non-invasive treatment for HO.
Bone metabolism is regulated by the local expression ratio of the osteoclast inducer Receptor Activator of Nuclear FactorκB Ligand (RANKL) to the native osteoclast inhibitor osteoprotegerin (OPG). Bone-forming osteoblasts express high RANKL/OPG early in their differentiation thus driving resorption, while mature, mineralizing, osteoblasts express low RANKL/OPG. We hypothesize that OPG expression is high in HO, blocking osteoclast formation and favoring maintenance of heterotopic bone. We propose that releasing the OPG blockade of osteoclast formation by systemic administration of anti-OPG function-blocking antibodies will allow osteoclast formation and prevent the development of HO. Importantly, due to high OPG expression, HO will be a selective target of this pro-resorptive approach.
Methods: We have employed a mouse model of HO with transgenic mice bearing a Cre-inducible constitutively-active ALK2 receptor construct (caALK2) (an R220A mutant of the BMP receptor ALK2) (Fukuda et. al., Genesis 2006). Expression of the caALK2 gene was induced by intramuscular (IM) injection of Cre-recombinase delivered by an adenoviral vector (Ad-Cre) (5 X 10E8 pfu). Following injection of Ad-Cre, animals reproducibly develop significant ectopic bone at the site of injection over a rapid 14 day time course. Commercially available anti-OPG antibodies were tested for their ability to block OPG inhibition of in vitro osteoclastogenesis using RAW264.7 cells cultured with 20ng/ml RANKL. Goat anti-OPG antibody from R&D Systems was chosen for high activity (#AF-459). Systemic treatment was by intraperitoneal (IP) injection of anti-OPG function blocking goat polyclonal antibody (10ug on days 2, 6, 10). HO production was demonstrated over time by a range-of-motion (ROM) assay (Yu et. al., Nat Med. 2008). MicroCT was used to quantify HO formation and contralateral native bone volume. Histology was used to characterize the HO formed and to identify tartrate-resistant acid phosphatase (TRACP) positive osteoclast cells. Immunohistochemistry was used to identify OPG producing cells. ELISA was used to measure TRACP5b, as a serum marker of resorptive activity.
Results: HO bone contained few osteoclast cells compared to long bones and HO tissues express high OPG as compared to normal skeletal sites. Systemic treatment with the inhibitory polyclonal antibody against OPG resulted in an increase in osteoclast numbers in HO bone with a statistically significant increase in serum TRAPC5b ($p<0.05$). Systemic treatment with anti-OPG also resulted in a statistically significant reduction of mineralized tissue (bone volume)in HO ($p=0.05$)(fig. 1) with a concomitant and statistically significant improvement in ROM at day 14 ($p=0.0038$)(fig. 2), compared to IgG treated controls. Notably, bone loss seen at ectopic sites was greater than loss at native skeletal sites in the anti-OPG treated animals.

Discussion: The finding that osteoclast numbers are low in HO bone as compared to skeletal sites indicates that osteoclast formation may indeed be inhibited at sites of HO. The demonstration of OPG expression in HO tissue supports our hypothesis that osteoclast differentiation is inhibited in HO by a mechanism which may involve OPG expression. Treatment with a polyclonal antibody resulted in increased osteoclastogenesis and resorption as demonstrated by the serum marker of resorption TRAPC5b. Increased osteoclastogenesis resulted in measurable and significant reduction of HO with an increase in range-of-motion.

Significance: These studies indicate that osteoclastic resorption is likely inhibited in HO by OPG expression and if OPG function is blocked, HO is reduced. Therefore, novel pro-resorptive therapies for HO may have a significant impact on the treatment of HO. In addition, the identification of more effective and specific OPG blocking reagents will enhance pro-resorptive therapies for HO.

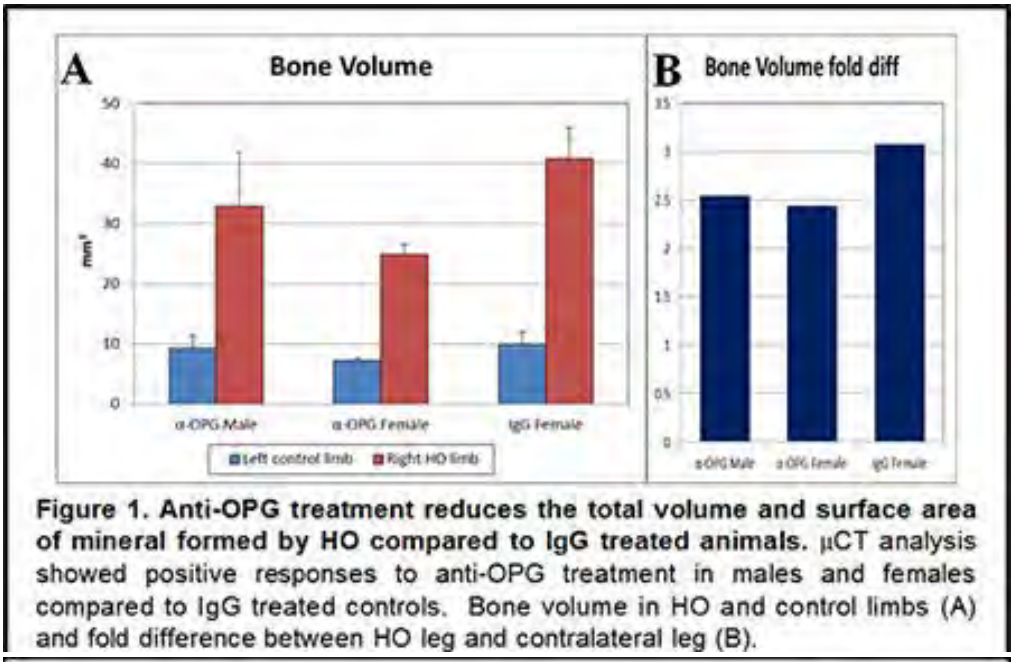

Figure 1. Anti-OPG treatment reduces the total volume and surface area of mineral formed by HO compared to IgG treated animals. µCT analysis showed positive responses to anti-OPG treatment in males and females compared to IgG treated controls. Bone volume in HO and control limbs (A) and fold difference between HO leg and contralateral leg (B).

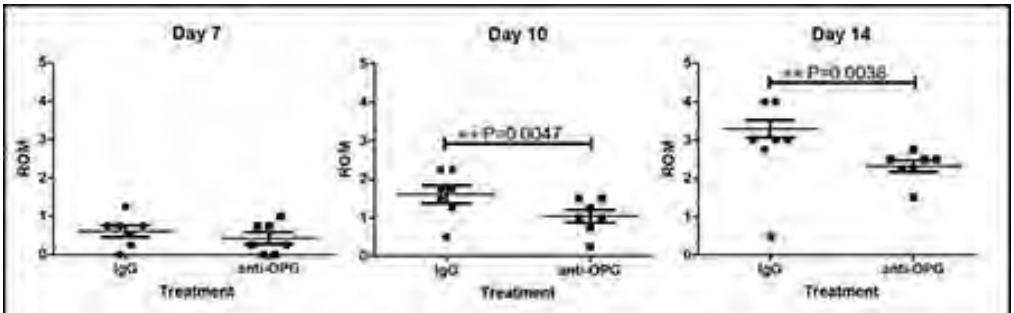

Figure 2. Treatment with function-blocking anti-OPG antibodies improves mobility. IP injection of anti-OPG leads to an improvement in the passive range-of-motion scores. Changes are significant at days 10 and 14. (*statistical outlier)